Villafranca, J. J., Ash, D. E., & Wedler, F. C. (1976) *Biochemistry 15*, 544–553.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wedler, F. C. (1974) *J. Biol. Chem. 249*, 5080–5087.
Wedler, F. C., & Boyer, P. D. (1972) *J. Biol. Chem. 247*, 984–992.
Wedler, F. C., & Horn, B. R. (1976) *J. Biol. Chem. 251*, 7530–7538.
Wedler, F. C., Willis, B. A., & Srubas, R. (1977) *Experientia 33*, 1016–1018.
Wedler, F. C., Shreve, D. S., Kenney, R. M., Ashour, A. E., Carfi, J., & Rhee, S. G. (1980) *J. Biol. Chem. 255*, 9507–9516.
Weisbrod, R. E., & Meister, A. (1973) *J. Biol. Chem. 248*, 3997–4002.
Wolodko, W. T., O'Conner, M. D., & Bridger, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2140–2144.

Affinity Labeling of a Guanosine 5′-Triphosphate Site of Glutamate Dehydrogenase by a Fluorescent Nucleotide Analogue, 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine†

Marlene A. Jacobson and Roberta F. Colman*

ABSTRACT: Bovine liver glutamate dehydrogenase is reversibly inhibited by the fluorescent nucleotide 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP) and reacts irreversibly with the corresponding affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. The enzyme is not inactivated by this reaction as measured in the absence of modifiers. However, a time-dependent increase is observed in the catalytic activity of the enzyme, when assayed in the presence of the allosteric inhibitor guanosine 5′-triphosphate (GTP). This change in inhibition by GTP allows determination of a rate constant for reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. A nonlinear dependence of the reaction rate on reagent concentration suggests a reversible binding prior to irreversible modification. The rate constant is unaffected by the substrate α-ketoglutarate or high concentrations of reduced diphosphopyridine nucleotide (DPNH) alone and is only slightly lowered by the activator adenosine 5′-diphosphate (ADP). A decrease in the rate constant is caused by added GTP or ϵATP, and a combination of GTP in the presence of reduced coenzyme provides complete protection. The compound 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-etheno[2-^{3}H]-adenosine was synthesized and used to show that 1.28 mol of 5′-(*p*-sulfonylbenzoyl)-1,N^6-ethenoadenosine/mol of subunit is incorporated at 100% change in sensitivity to GTP inhibition. As compared to native glutamate dehydrogenase, the modified enzyme exhibits a decreased affinity for and diminished maximum inhibition by saturating concentrations of GTP, a decreased maximum extent of activation with no change in affinity for ADP, and a normal ability to be inhibited by high DPNH concentrations. In contrast to the 2 mol of GTP bound by native enzyme, only 1 mol of GTP is bound per peptide chain of the modified enzyme in the absence or presence of DPNH. This implies that one of the natural GTP sites is eliminated as a result of reaction with 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. This study indicates that reaction occurs at a GTP site and suggests that alteration of the N-1 and 6-NH_2 positions of the adenine ring leads to recognition by glutamate dehydrogenase as an inhibitory guanine nucleotide.

The fluorescent nucleotides 1,N^6-ethenoadenosine triphosphate and 1,N^6-ethenoadenosine diphosphate have been widely utilized in probing the environment of binding sites in proteins (Secrist et al., 1972; Yanagida, 1981). These compounds possess two important properties that can be useful in studies of protein structure: first, the fluorescent properties can be employed in the measurement of the distance between sites on a protein by energy transfer (Brand & Witholt, 1967; Horton & Koshland, 1967; Stryer, 1978); second, since the N-1 and 6-NH_2 purine positions are altered by the etheno bridge in the 1,N^6-ethenoadenine moiety of these nucleotides, the importance of these positions in determining the specificity of binding of these nucleotides to particular enzymes can be evaluated.

The activity of the allosteric enzyme bovine liver glutamate dehydrogenase [L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3] is modulated by GTP,[1] which inhibits, ADP, which activates, and DPNH, which inhibits at high concentrations by binding at a site distinct from the catalytic site (Goldin & Frieden, 1972). Glutamate dehydrogenase is composed of six identical subunits with several nucleotide sites per subunit, including a site for ADP, two for GTP, and two for DPNH (one catalytic and one regulatory) (Pantaloni & Dessen, 1969; Goldin & Frieden, 1972; Pal & Colman, 1979). The number of GTP binding sites observed depends on the presence of the reduced coenzyme: in the absence of DPNH, native enzyme has one GTP binding site, whereas in the presence of DPNH, two GTP binding sites are observed that differ in affinity for the guanine nucleotide (Pal & Colman, 1979).

† From the Department of Chemistry, University of Delaware, Newark, Delaware 19711. *Received September 16, 1981.* This work was supported by U.S. Public Health Service Grant GM 21200.

[1] Abbreviations: 5′-FSBϵA, 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine; ϵATP, 1,N^6-ethenoadenosine 5′-triphosphate; ϵADP, 1,N^6-ethenoadenosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; DPNH, reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; TPNH, reduced triphosphopyridine nucleotide.

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Several chemical modification studies have been aimed at identifying the amino acid residues in the substrate and regulatory sites of glutamate dehydrogenase (Goldin & Frieden, 1972). The use of nonspecific reagents has complicated the interpretation of these studies because of modification of multiple amino acids and/or because of alteration of more than one of the catalytic and regulatory functions of the enzyme. In the attempt to separately and specifically modify the various regulatory sites on this enzyme, its reactions with several purine nucleotide affinty labels have been studied. The adenosine analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine has been shown to specifically modify the DPNH regulatory site (Pal et al., 1975a; Saradambal et al., 1981). Another adenosine analogue, 3′-[*p*-(fluorosulfonyl)benzoyl]adenosine (Pal et al., 1975b), appears to modify the DPNH regulatory site as well but produces an enzyme that is also altered in its response to ADP activation. In contrast, the guanosine analogue 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine acts as an affinity label in its reaction at one of the GTP sites of glutamate dehydrogenase (Pal & Colman, 1979).

The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine has recently been synthesized (Likos & Colman, 1981). In addition to its fluorescent properties (emission maximum at 412 nm), this compound has the potential for reacting covalently with nucleophilic groups in proteins. Glutamate dehydrogenase has been reported to be reversibly inhibited by 1,N^6-ethenoadenosine triphosphate (Lascu et al., 1977), although the relationship between the site occupied by ϵATP and the other nucleotides was not defined. This study demonstrates the specific covalent reaction of 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine with glutamate dehydrogenase. To provide the essential background information necessary to use this analogue as a covalent fluorescent probe of glutamate dehydrogenase, we have examined the functional site of modification and the effects of structural modification of the purine moiety on the recognition of the nucleotide by the enzyme. A preliminary account of this work has been presented (Jacobson & Colman, 1981).

Experimental Procedures

Materials. Bovine liver glutamate dehydrogenase, purchased as a crystalline suspension in ammonium sulfate from Boehringer Mannheim Corp., was dialyzed for 16 h at 4 °C against two changes of 0.1 M potassium phosphate buffer, pH 7.1. The dialyzed material was centrifuged at 4 °C for 20 min at 15000 rpm to remove precipitated denatured protein. The enzyme concentration was determined with the value $E^{1\%}_{279}$ = 9.7 (Olson & Anfinsen, 1952); the ratio A_{280}/A_{260} was 1.9. The enzyme was stored in aliquots at −85 °C. A molecular weight of 56 100 for identical peptide chains was used in the calculations (Smith et al., 1970).

The compound 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (5′-FSBϵA) was prepared by the method of Likos & Colman (1981) with the following modifications: ethenoadenosine (0.31 mmol) was dissolved in 2 mL of hexamethylphosphoramide with warming before the addition of *p*-(fluorosulfonyl)benzoyl chloride; the product, 5′-FSBϵA, was purified on 1500-μm silica gel plates (Analtech, Inc.). Radioactive ethenoadenosine hydrochloride was synthesized by the addition of 5 mCi of [2-^{3}H]adenosine (New England Nuclear Corp.) to a solution of 650 mg (2.43 mmol) of nonradioactive adenosine in 25 mL of aqueous chloroacetaldehyde. Preparation of chloroacetaldehyde and ethenoadenosine was in accordance with the procedure of Secrist et al. (1972). Radioactive [2-^{3}H]-5′-FSBϵA was synthesized from the prepared etheno[2-^{3}H]adenosine, following the procedure of Likos & Colman (1981) with the same modifications described above. Solutions of 5′-FSBϵA were prepared by dissolving the reagent in distilled dimethylformamide, and concentrations were determined spectrophotometrically at 275 nm (ϵ_{275} = 7632 M^{-1} cm^{-1}, in ethanol).

[U-^{14}C]GTP was purchased from New England Nuclear Corp. All coenzymes and purine nucleotides, as well as EDTA, Tris base, and dithiothreitol, were purchased from Sigma Chemical Co. All other chemicals were reagent grade.

Enzymatic Assay. Glutamate dehydrogenase activity was assayed spectrophotometrically at 340 nm by measuring the oxidation of reduced coenzyme at 25 °C in Tris–0.01 M acetate buffer, pH 8 (containing 10 μM EDTA), with a Gilford Model 240 spectrophotometer equipped with an expanded-scale recorder (0–0.1 full scale). For the standard assay, the substrate concentrations used were 5 mM α-ketoglutarate, 50 mM ammonium chloride, and 100 μM DPNH. The total volume of the assay solution was 1.0 mL. When the activity was measured in the presence of a constant concentration of the inhibitor GTP, the nucleotide concentration was 1.0 μM. Conditions used for testing the effects of varying concentrations of regulatory compounds, such as ADP or GTP, are indicated under Results.

Reaction of 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine with Glutamate Dehydrogenase. Glutamate dehydrogenase (0.25–1 mg/mL) was incubated with 5′-FSBϵA (0.5–2.1 mM) at 30 °C in 0.01 M sodium barbital buffer (pH 8) containing 0.2 M KCl and 10% dimethylformamide. Dimethylformamide was required to maintain the solubility of the reagent and had no effect on the stability or activity of the enzyme as determined from a control that consisted of enzyme incubated under the same conditions but in the absence of 5′-FSBϵA. Aliquots were withdrawn at given time intervals, diluted at least 21-fold in Tris–0.1 M acetate (pH 8) at 0 °C, and assayed in the absence or presence of GTP, with 30 or 60 μL, respectively, in a total volume of 1.0 mL. The overall dilution of the reaction mixture in the assay was at least 1:350. Data presented have been normalized for any differences in protein concentration used in the assay.

Determination of the Rate Constant for Decomposition of 5′-FSBϵA. The rate constant for decomposition of 5′-FSBϵA was measured by incubating the reagent (1.2 mM) in the absence of enzyme under conditions similar to those described for reaction, i.e., in 0.02 M potassium barbital buffer, pH 8, containing 0.2 M KCl and 15% dimethylformamide. The decomposition of 5′-FSBϵA was monitored at 25 °C by measuring the amount of fluoride released as a function of time with a Beckman pH meter equipped with an Orion combination fluoride electrode. The rate constant for decomposition of 5′-FSBϵA was calculated from a semilogarithmic plot of $[5'\text{-FSB}\epsilon\text{A}]_t/[5'\text{-FSB}\epsilon\text{A}]_0$ as a function of time.

Incorporation of 5′-(Sulfonylbenzoyl)-1,N^6-ethenoadenosine by Glutamate Dehydrogenase. Enzyme (0.25 mg/mL) was incubated with 1.4 mM [2-^{3}H]-5′-FSBϵA under the conditions described above. At a given extent of reaction, enzyme was separated from excess reagent by the column–centrifuge procedure described by Penefsky (1979). Columns were prepared in a 5-mL disposable syringe barrel filled with Sephadex G-50, 80 mesh, equilibrated with 0.1 M potassium phosphate buffer, pH 7.1. The column was supported within the barrel by a small glass wool plug. The amount of reagent incorporated into the protein was determined by measuring the radioactivity in two 100-μL aliquots from the eluate obtained by centrifugation in a clinical centrifuge; the aliquots

were measured in ACS scintillation liquid (Amersham) with a Packard TriCarb liquid scintillation counter, Model 3330. Protein concentration was determined with the Bio-Rad Protein Assay, which is based on the method of Bradford (1976). Native glutamate dehydrogenase was used to establish a standard curve. Two successive centrifuge columns were required for complete removal of noncovalently bound reagent.

Preparation of Modified Enzyme. Glutamate dehydrogenase (1 mg/mL) was incubated with 1.4 mM 5′-FSBεA under the conditions described above. The total concentration of phosphate was 1.9 mM in the reaction mixture. After 120 min, a second aliquot of 5′-FSBεA was added so that the final concentration of new reagent was 0.7 mM. One hour after the second addition of reagent, the modified enzyme was isolated by the column–centrifuge method described above except that the Sephadex G-50, 80 mesh, was equilibrated with Tris–0.05 M acetate buffer (pH 7.1) containing 10 mM potassium phosphate and 100 μM EDTA.

GTP Binding Studies. Binding of [U-^{14}C]GTP to native and modified enzymes was measured by an ultrafiltration technique (Colman & Foster, 1970) at 25 °C in Tris–0.044 M acetate buffer (pH 7.1) containing 8.75 mM potassium phosphate and 87.5 μM EDTA. An Amicon Model 10-PA ultrafiltration cell was assembled with a PM-10, 25-mm membrane to separate free ligand from enzyme-bound ligand. Previous determinations (Pal & Colman, 1979) indicate that retention of radioactivity by the membrane was less than 1% in the absence of enzyme. Both free and total ligand concentrations were determined from the specific activity of [U-^{14}C]GTP with a liquid scintillation counter. The concentration of bound ligand was determined from the difference between the concentrations of total and free ligand.

Results

Effect of 1,N^6-Etheno-ATP and 1,N^6-Etheno-ADP on the Catalytic Activity of Glutamate Dehydrogenase. Glutamate dehydrogenase is reversibly inhibited by 1,N^6-etheno-ATP (εATP) as assayed in Tris–0.01 M acetate buffer, pH 8, at 25 °C with 100 μM DPNH as the coenzyme. The activity of the enzyme was measured in the presence of εATP over a concentration range of 1 μM–3.3 mM. The dissociation constant for an allosteric inhibitor of glutamate dehydrogenase has been shown to be numerically equal to the inhibitor concentration at which the velocity equals

$$(1/2)(V + V_I) \qquad (1)$$

where V and V_I are the maximum velocities in the absence and presence of saturating concentrations of the inhibitor, respectively (Frieden, 1963). With the use of eq 1, a dissociation constant for the enzyme–εATP complex was determined as 22 μM. At saturating concentrations of εATP, a maximum extent of inhibition of 91% was observed.

Glutamate dehydrogenase is also reversibly inhibited by 1,N^6-etheno-ADP (εADP) when assayed in Tris–acetate buffer, as above. This observation contrasts with the previous report (Dieter et al., 1974) that εADP had no effect on the catalytic activity of the enzyme when measured in 0.067 M phosphate buffer, pH 7.6. The enzymatic activity in the presence of εADP has now been measured over a concentration range of 5 μM–3.3 mM. With the use of eq 1, the dissociation constant for the enzyme–εADP complex was determined to be 225 μM. At saturating concentrations of εADP, a maximum extent of inhibition of 87% was observed.

The inhibitory effects of εATP and εADP can be compared to the effects observed with the guanine nucleotide inhibitors GTP and GDP. The inhibition by εATP is similar to that exhibited by GTP in that the maximum extent of inhibition compares reasonably well (96% maximum inhibition by GTP). In addition, the weaker binding of εADP as compared to that of εATP is similar to the weaker binding of GDP as compared to that of GTP (Frieden, 1963).

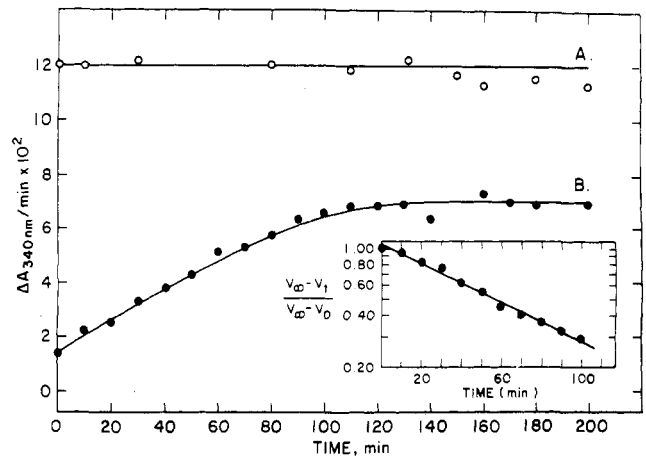

FIGURE 1: Reaction of 1.4 mM 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine with glutamate dehydrogenase. The enzyme (0.25 mg/mL) was incubated as described under Experimental Procedures and assayed (A) in the absence and (B) in the presence of 1 μM GTP. Total phosphate concentration in the reaction mixture was 2.3 mM. (Inset) Determination of the pseudo-first-order rate constant from the increase in enzyme activity assayed in the presence of GTP. The increase in activity as a function of time is expressed as $(V_\infty - V_t)/(V_\infty - V_0)$ where V_t and V_0 are the velocities at a given time and at time zero; V_∞ is the constant velocity reached at complete reaction determined by the successive addition experiments.

Reaction of Glutamate Dehydrogenase with 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine. Incubation of glutamate dehydrogenase with 1.4 mM 5′-FSBεA for 200 min produces no change in the maximum velocity when assayed in the absence of modifiers (Figure 1, curve A). Since the enzyme is not inactivated by covalent reaction, it appears that 5′-FSBεA does not react at the active site. However, a time-dependent increase in the activity of the enzyme as assayed in the presence of a constant concentration of the inhibitor GTP is observed. The rate of reaction of 5′-FSBεA with the enzyme can be monitored by this observed time-dependent desensitization to GTP inhibition (Figure 1, curve B).

To determine if the plateau observed in curve B of Figure 1 is due to the attainment of the end point for the reaction or alternatively can be attributed to reagent decomposition, we conducted the following experiments. Glutamate dehydrogenase (0.25 mg/mL) was incubated with 0.7 mM 5′-FSBεA. After 155 min, a second addition of reagent was made such that the concentration of newly added reagent was 0.7 mM in the reaction mixture (Figure 2, point A). The velocity measured in the presence of GTP increased approximately 1.8-fold by 145 min after the second addition. At this time (Figure 2, point B), a third addition of reagent was made so that the final concentration of newly added reagent was again 0.7 mM. As indicated in Figure 2, the activity of the enzyme as assayed in the presence of GTP did not appear to change appreciably after this third addition of reagent, suggesting that the end point for the reaction had been reached. Thus, the plateau observed in curve B of Figure 1 must represent an apparent end point (V_∞') due to reagent decomposition. The activity of the enzyme, assayed in the absence of GTP, decreases slightly after the third addition. A reaction mixture consisting of glutamate dehydrogenase (0.25 or 1 mg/mL) was incubated initially with 1.4 mM FSBεA, followed by the addition at 120 min of reagent to produce an additional con-

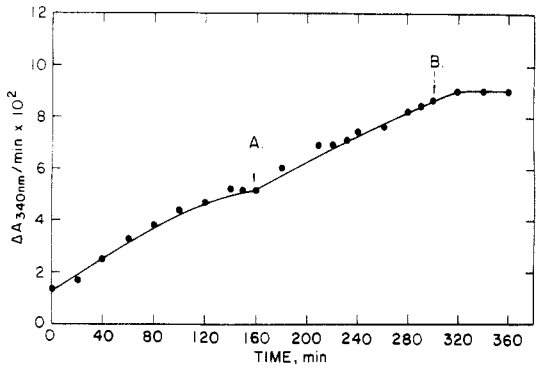

FIGURE 2: Reaction of glutamate dehydrogenase with successive additions of 5′-FSBϵA. The enzyme (0.25 mg/mL) was incubated as described under Experimental Procedures and assayed in the presence of 1 μM GTP. Reaction is initiated with 0.7 mM 5′-FSBϵA. Second (A) and third (B) additions of 5′-FSBϵA were made at 155 and 300 min, respectively, as described under Results. Initially, the percent of dimethylformamide was 7.5 in the reaction mixture; the final concentration after the third addition was 12%.

centration of 0.7 mM in the reaction mixture; after 60 min, the same end point was attained as in the case of three successive additions of 0.7 mM 5′-FSBϵA. This value of $\Delta A_{340}/\text{min} = 0.090$ was subsequently used for the end point (V_∞) of the reaction. The maximum velocity measured in the presence of 1 μM GTP at the end point is 75% of the maximum velocity measured in the absence of GTP. With the use of this end point, the pseudo-first-order rate constant was determined from a semilogarithmic plot (Figure 1, inset) of the time-dependent increase in the activity of glutamate dehydrogenase as assayed in the presence of 1 μM GTP.

The observation has been made with 5′-FSBϵA (Likos & Colman, 1981) and with other fluorosulfonyl derivatives (Togashi & Reisler, 1981; Williamson, 1981; Annamalai & Colman, 1981; Tomich et al., 1981) that addition of dithiothreitol reverses the reaction of the analogue with certain enzymes. This observation has been interpreted to indicate the involvement of a thiolsulfonate linkage between the reagent and a cysteine residue at the modification site. Addition of either 20 or 100 mM dithiothreitol to a reaction mixture of glutamate dehydrogenase and 5′-FSBϵA in which the enzyme was modified 67% did not reverse the desensitization of the enzyme to GTP nor did it affect the activity of the enzyme as assayed in the absence of GTP, suggesting that the modification of cysteine residues is not responsible for the 5′-FSBϵA-mediated decrease in response of the enzyme of GTP.

Kinetics of Reaction of Glutamate Dehydrogenase with 5′-[p-(Fluorosulfonyl)benzoyl]-1,N⁶-ethenoadenosine. The pseudo-first-order initial rate constant for reaction of glutamate dehydrogenase with 5′-FSBϵA was measured as a function of concentration over the range of 0.5–2.1 mM 5′-FSBϵA. The observed rate constants exhibit a nonlinear dependence on 5′-FSBϵA concentration. This observation suggests that the reagent I binds reversibly to the enzyme prior to irreversible covalent modification to yield modified enzyme (EI′) as follows:

$$E + I \underset{}{\overset{K_D}{\rightleftharpoons}} EI \xrightarrow{k_1} EI' \qquad (2)$$

The observed rate constant for modification can be expressed as

$$k_{obsd} = \frac{k_1}{1 + (K_D/[I])} \qquad (3)$$

where k_{obsd} is the apparent rate constant observed at a particular concentration of 5′-FSBϵA, k_1 is the maximal rate for

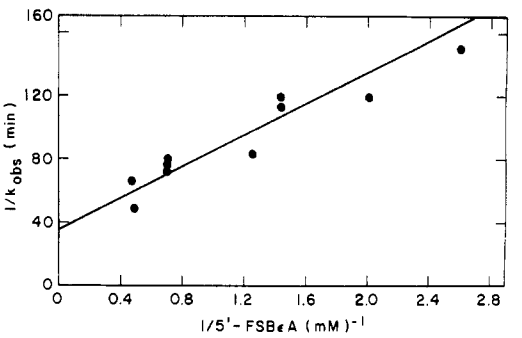

FIGURE 3: Dependence of the pseudo-first-order rate constant k_{obsd} on the concentration of 5′-FSBϵA. Glutamate dehydrogenase (0.25 mg/mL) was incubated with varying concentrations of 5′-FSBϵA as described under Experimental Procedures. Rate constants were calculated as described under Results.

covalent modification observable at infinite reagent concentration, and K_D is the dissociation constant of the enzyme–5′-FSBϵA complex. The reciprocal form of eq 3 is

$$\frac{1}{k_{obsd}} = \frac{1}{k_1} + \frac{K_D}{k_1}\frac{1}{[I]} \qquad (4)$$

From a double-reciprocal plot of $1/k_{obsd}$ vs. $1/[5'\text{-FSB}\epsilon\text{A}]$ (Figure 3), a dissociation constant, $K_D = 1.4$ mM, and maximum rate constant, $k_1 = 0.028$ min^{-1}, can be calculated.

It was noticed that the apparent end point for the reaction seemed to increase as the concentration of 5′-FSBϵA was increased. The end point for the reaction of glutamate dehydrogenase can be predicted by analysis of two competing reactions: that between the enzyme and reagent I (eq 2) and that of the decomposition of the reagent due to fluoride release according to

$$I \xrightarrow{k_2} I + F^- \qquad (5)$$

where k_2 is the rate constant for the decomposition of 5′-FSBϵA. If a steady-state approximation is made for EI and a time-dependent depletion of I is considered, the following expression can be derived for the rate of change of E, the total amount of enzyme:

$$-\frac{dE}{dt} = \frac{k_1[E][I_0]e^{-k_2t}}{K_D + [I_0]e^{-k_2t}} \qquad (6)$$

The integrated form of this equation is

$$\ln\frac{[E_0]}{[E_t]} = \frac{k_1}{k_2}\left[k_2t + \ln\left(\frac{K_D}{[I_0]} + 1\right) - \ln\left(\frac{K_D}{[I_0]}e^{k_2t} + 1\right)\right] \qquad (7)$$

where experimentally the ratio of total enzyme to unmodified enzyme at time t (E_0/E_t) is measured by $(V_\infty - V_0)/(V_\infty - V_t)$. As time increases, a limit is approached and eq 7 becomes

$$\ln\frac{[E_0]}{[E_\infty]} = \frac{k_1}{k_2}\left[\ln\left(\frac{K_D}{[I_0]} + 1\right) - \ln\frac{K_D}{[I_0]}\right] \qquad (8)$$

The constants are the same as defined above. A value of $k_2 = 0.0163$ min^{-1} was determined for the rate of decomposition of the reagent measured by the rate of fluoride release. The predicted extent of reaction, defined by $1 - ([E_\infty]/[E_0])$ in terms of the amount of modified enzyme, can be calculated directly from the experimental data. Comparison of the predicted extent of reaction from eq 8 and the observed extent

Table I: Comparison of Predicted and Observed Extent of Reaction of 5′-FSBεA with Glutamate Dehydrogenase[a]

5′-FSBεA (mM)	extent of reaction (%)	
	predicted	observed
0.38	34	28
0.50	40	37
0.70	50	45
0.80	54	50
1.4	70	61
2.06	79	74

[a] Glutamate dehydrogenase (0.25 mg/mL) was incubated with varying concentrations of 5′-FSBεA as described under Experimental Procedures. The observed extent of reaction is defined as $(V_\infty' - V_0)/(V_\infty - V_0)$, where V_∞' is the apparent end point observed in the presence of 1 μM GTP for a particular concentration of 5′-FSBεA; V_0 and V_∞ are, respectively, the velocities measured in the presence of 1 μM GTP at time zero and when the reaction is complete as determined by successive additions. The predicted extent of reaction was determined with eq 8, with K_D and k_1 determined from Figure 3 and $k_2 = 0.0163$ min^{-1} measured by fluoride release with a fluoride electrode as described under Experimental Procedures.

of reaction over a range of 5′-FSBεA concentrations is shown in Table I, indicating reasonable agreement. The experimental data are thus consistent with the model represented by eq 2 and 5 and imply that complete reaction cannot be attained with a single addition of 1.4 mM 5′-FSBεA; successive additions of reagent appear to be required for total modification of the enzyme.

The effects of added substrates and regulatory compounds on the rate of reaction of glutamate dehydrogenase with 1.4 mM 5′-FSBεA are shown in Table II. The rate constant is unaffected by the inclusion in the reaction mixture of the substrate α-ketoglutarate or of the coenzyme DPNH when these are present at concentrations high relative to their known dissociation constants (Goldin & Frieden, 1972). This indicates that modification by 5′-FSBεA does not occur at the active site. Glutamate dehydrogenase is inhibited by high concentrations of DPNH, which binds at a regulatory site distinct from the catalytic site (Frieden, 1959). At saturating DPNH concentrations for this regulatory site, the rate of reaction was unaffected. The activator ADP included either alone or in the presence of DPNH lowers the rate constant only slightly. Since the activator was present in a concentration much higher than its K_D value (Frieden, 1963), it is considered that the change in the rate constant is due to an indirect effect of ADP on modification of a site distinct from the ADP site.

The rate constant for modification by 5′-FSBεA is decreased with increasing concentrations of GTP alone but is most markedly altered by combinations of GTP and DPNH. Complete protection is provided by saturating concentrations of these ligands (i.e., 25 or 100 μM GTP in the presence of 100 μM DPNH). In the presence of a protecting ligand (L) the enzyme can form either an irreversibly modified enzyme or a reversible enzyme–ligand complex exhibiting a dissociation constant, K_L. On the assumption that the ligand competes functionally with the irreversible reactant, the observed rate constant for modification can be expressed as

$$k_{obsd} = \frac{k_1}{1 + \frac{K_D}{[I]}\left(1 + \frac{[L]}{K_L}\right)} \quad (9)$$

where k_1 is the rate constant for modification in the absence of ligands, [L] is the concentration of protecting ligand, K_L is the dissociation constant for enzyme–ligand complex, [I] is the concentration of 5′-FSBεA, and K_D is the dissociation constant for enzyme–reagent complex. Calculation of dissociation constants from the data of Table II indicates for GTP alone values of 300–100 μM, which are considerably higher than the value obtained from direct binding studies, $K_D = 28.6$ μM.

Table II: Effect of Substrates and Modifiers on the Rate of Reaction of 5′-[*p*-(Fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine with Glutamate Dehydrogenase[a]

additions to reaction mixture	k_{obsd} (min^{-1}) × 10^3
none	13.2
20 mM α-ketoglutarate	12.7
100 μM DPNH	12.9
3 mM DPNH	13.2
2 mM ADP	10.7
2 mM ADP + 100 μM DPNH	7.7
10 μM GTP	13.3
100 μM GTP	12.0
200 μM GTP	8.4
400 μM GTP	4.7
2 μM GTP + 100 μM DPNH	11.3
5 μM GTP + 100 μM DPNH	6.5
10 μM GTP + 100 μM DPNH	1.0
25 μM GTP + 100 μM DPNH	no reaction
100 μM GTP + 100 μM DPNH	no reaction
100 μM εATP	9.0
3.3 mM εATP	6.6
3.3 mM εATP + 100 μM DPNH	2.6

[a] Glutamate dehydrogenase (0.25 mg/mL) was incubated with 5′-FSBεA (1.4 mM) at 30 °C in 0.01 M sodium barbital buffer (pH 8.0) containing 0.2 M KCl and 10% dimethylformamide. The pseudo-first-order rate constants for the reaction were determined in accordance with the inset of Figure 1 and Results.

GTP has been shown to bind more tightly to glutamate dehydrogenase in the presence of reduced coenzyme and to exhibit an apparent positive cooperativity among binding sites; i.e., the dissociation constant for the enzyme–GTP complex appears to decrease with increasing extent of saturation of the enzyme with GTP (Frieden & Colman, 1967). From the rate constants shown in Table II, a systematic decrease is observed in the dissociation constant for GTP measured in the presence of DPNH as the GTP concentration is increased: $K_D = 4.2$ μM at 2 μM GTP, $K_D = 2.2$ μM at 5 μM GTP, and $K_D = 0.39$ μM at 10 μM GTP. The characteristics of GTP in protecting glutamate dehydrogenase against modification by 5′-FSBεA are thus consistent with the previously observed binding behavior for GTP in the presence of DPNH.

A 2-fold protection is observed with the inclusion in the reaction mixture of 3.3 mM εATP. The protection is increased 5-fold when 3.3 mM εATP is combined with DPNH. The effect of εATP appears to mimic that observed for GTP, except that the dissociation constant for the enzyme–εATP complex appears to be higher. These results are consistent with the reaction of 5′-FSBεA with glutamate dehydrogenase occurring at a GTP site.

Incorporation of 5′-[p-(Fluorosulfonyl)benzoyl]-1,N^6-etheno[2-^{3}H]adenosine by Glutamate Dehydrogenase. The radioactive compound [2-^{3}H]-5′-FSBεA was used to determine the amount of reagent incorporated by glutamate dehydrogenase by the column–centrifuge method described under Experimental Procedures. The extent of covalent incorporation of 1.4 mM [2-^{3}H]-5′-FSBεA into glutamate dehydrogenase (Figure 4) increases with time and is directly proportional to the percent decrease in GTP inhibition, extrapolating to 1.28 mol of [2-^{3}H]-5′-SBεA/mol of subunit at 100% change in sensitivity to GTP inhibition.

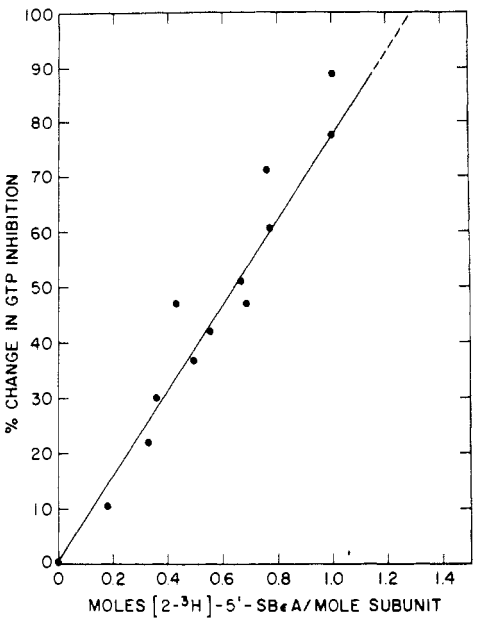

FIGURE 4: Incorporation of [2-^{3}H]-5′-SBϵA as a function of percent change in GTP inhibition. Glutamate dehydrogenase (0.25 mg/mL) was incubated with [2-^{3}H]-5′-FSBϵA (1.4 mM); points above 62% change in GTP inhibition were obtained by successive additions of 1.4 and 0.7 mM 5′-FSBϵA as described under Results; aliquots were withdrawn at various times and assayed in the presence of 1 μM GTP. Excess reagent was removed by column centrifugation as described under Experimental Procedures. The percent change in GTP inhibition is defined by $(V_t - V_0)/(V_\infty - V_0) \times 100$ where V_t is the enzymatic velocity measured in the presence of GTP at a particular time and V_0 and V_∞ are the velocities measured under the same conditions at time zero and at complete reaction, respectively.

Glutamate dehydrogenase incubated with 1.4 mM [2-^{3}H]-5′-FSBϵA for 1 h in the presence of 100 μM GTP and 100 μM DPNH incorporated 0.26 mol of [2-^{3}H]-5′-SBϵA/mol of subunit; however, no loss in sensitivity to GTP inhibition was observed. It seems likely that the limited modification observed under these conditions (i.e., complete protection) is the result of modification of sites other than those responsible for the desensitization to GTP inhibition. To determine if the slight protection observed in the presence of 2 mM ADP and 100 μM DPNH is correlated with a decrease in the amount of reagent incorporated, we incubated glutamate dehydrogenase with 1.4 mM [2-^{3}H]-5′-FSBϵA for 100 min in the presence of these ligands. The amount of incorporation measured (0.51 mol of [2-^{3}H]-5′-SBϵA/mol of subunit at 42% change in GTP inhibition) falls on the line determined for enzyme reacting in the absence of ligands. In the presence of ADP and DPNH, the enzyme is not modified as extensively as it is in the absence of these ligands; however, the decrease in the incorporation observed occurs only to the extent that ADP and DPNH decrease the change in inhibition by GTP.

Dithiothreitol has been reported to decrease the incorporation of 5′-SBϵA into pyruvate kinase (Likos & Colman, 1981). Dithiothreitol (100 mM) was added to a reaction mixture containing 5′-FSBϵA and glutamate dehydrogenase, which exhibited a 69% change in GTP inhibition. Incubation was continued for an additional 2 h after which the amount of incorporation was determined to be 0.81 mol of [2-^{3}H]-5′-SBϵA. Within experimental error, this point falls on the line shown in Figure 4. Thus, in contrast to the reaction of 5′-FSBϵA with pyruvate kinase, the extent of incorporation as well as the rate of reaction is not affected by the addition of dithiothreitol.

Kinetic Properties of Modified Enzyme. Modified enzyme was prepared from enzyme incubated at 1 mg/mL with 5′-FSBϵA as described under Experimental Procedures. The enzyme contained 1.28 mol of 5′-SBϵA/mol of subunit and exhibited 84% of the maximum change in GTP inhibition. The catalytic and regulatory properties of the modified enzyme were compared with those of native enzyme to examine the effect of covalent reaction and ascertain the site of modification.

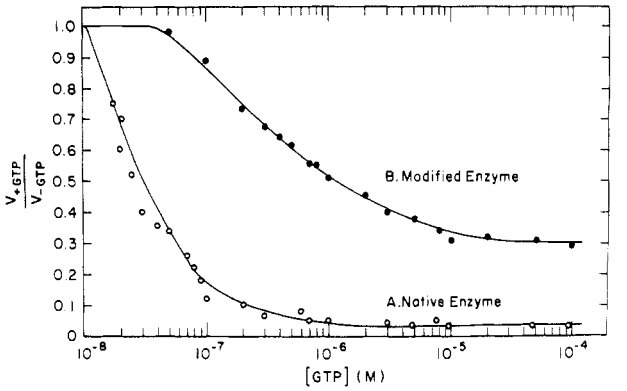

FIGURE 5: Ratio of maximum velocities in the presence and absence of GTP for native and modified enzymes. The velocities were measured for (A) native and (B) modified enzymes as described under Experimental Procedures with 100 μM DPNH as the coenzyme. Modified enzyme contains 1.28 mol of 5′-SBϵA incorporated/subunit.

To determine if there is any alteration in the affinity of the modified enzyme for GTP, we measured the velocities of native and modified enzymes as a function of GTP concentration and compared them as shown in Figure 5. The dissociation constant for the enzyme–GTP complex can be determined with eq 1. Curve A of Figure 5 indicates a dissociation constant of 0.025 μM for native enzyme and a maximum extent of inhibition of 96%. The K_{GTP} value is somewhat lower than that previously reported for native enzyme (Frieden, 1963) but was determined on the same lot of native enzyme used in the preparation of modified enzyme. The dissociation constant of the modified enzyme for GTP (Figure 5, curve B) is 0.37 μM, approximately 15 times higher than that for native enzyme. In comparison to the 96% inhibition for native enzyme at saturating concentrations of GTP, the maximum extent of inhibition is 70%. The K_{GTP} for native and modified enzyme was also measured with 100 μM TPNH as the coenzyme. Under these conditions, the native enzyme exhibits a dissociation constant for GTP of 0.15 μM and a 96% maximum extent of inhibition at saturating concentrations. In comparison, the K_{GTP} for modified enzyme is 1.3 μM with 74% maximum extent of inhibition at saturating concentrations. These values are consistent with an approximately 5-fold higher value for K_{GTP} when measured in the presence of TPNH as compared to DPNH (Goldin & Frieden, 1972). It is apparent that the sensitivity of glutamate dehydrogenase to GTP inhibition is decreased (when assayed with either DPNH or TPNH as the coenzyme) but not eliminated by reaction of 5′-FSBϵA. Glutamate dehydrogenase isolated after modification in the presence of 100 μM GTP and 100 μM DPNH exhibits the same concentration dependence for inhibition by GTP as does native enzyme: the curve generated is superimposable on curve A of Figure 5, indicating complete protection against modification of the GTP site.

The velocities of both native and modified enzymes were measured as a function of ADP concentration and are compared in Figure 6. The dissociation constant for the enzyme–ADP complex has been shown to be numerically equal to the concentration of ADP at which the velocity equals

$$(1/2)(V + V_A) \qquad (10)$$

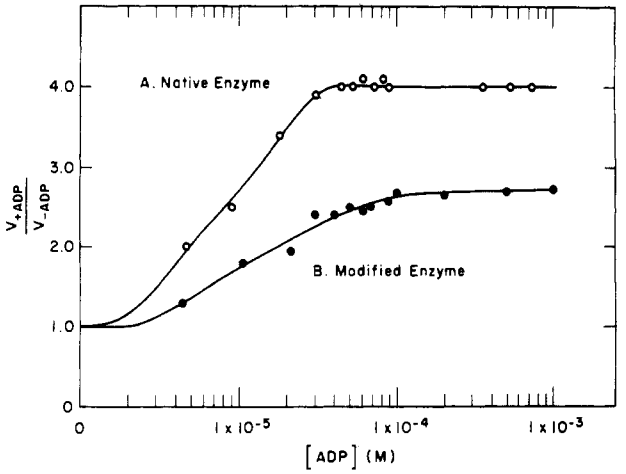

FIGURE 6: Ratio of maximum velocities in the presence and absence of ADP as a function of ADP concentration for native and modified enzymes. The velocities were measured for (A) native and (B) modified enzyme as described under Experimental Procedures with 100 μM TPNH as the coenzyme. Modified enzyme contains 1.28 mol of 5'-SBϵA incorporated/subunit.

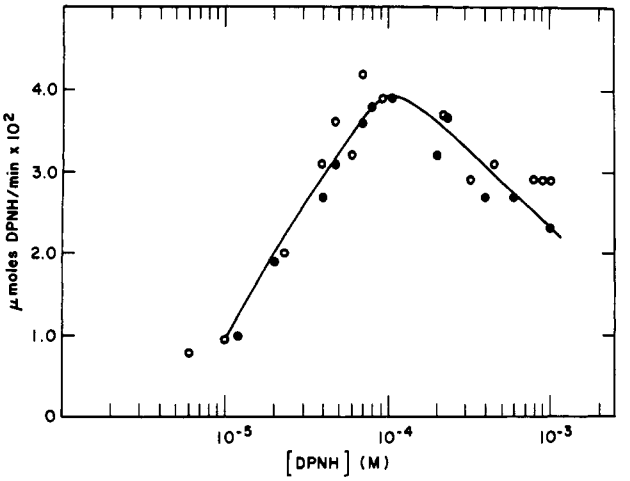

FIGURE 7: Initial velocity as a function of DPNH concentration for native (O) and modified enzyme (●) with 1.28 mol of 5'-SBϵA incorporated/subunit. At DPNH concentrations up to 2×10^{-4} M, velocity measurements were made at 340 nm as described under Experimental Procedures with the value of $\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1} for DPNH. At higher DPNH concentrations, measurements were made at 375 nm with the value of $\epsilon_{375} = 1.85 \times 10^3$ M^{-1} cm^{-1} for DPNH in calculating the rates in units of micromoles of DPNH per minute.

where V and V_A are the maximum velocities in the absence and presence of saturating concentrations of the activator, respectively (Frieden, 1963). As indicated in Figure 6, native and modified enzymes show a similar affinity for ADP: modified enzyme has a dissociation constant of 11 μM, whereas that for native enzyme is 9 μM. However, the maximum extent of activation is decreased significantly to 2.5-fold in the modified enzyme as compared with a 4-fold activation observed for native enzyme. The dissociation constant for the enzyme–ADP complex was also measured with 100 μM DPNH as the coenzyme in the enzymatic assay. Both modified and native enzymes exhibited similar dissociation constants of 55 and 40 μM, respectively. A significant decrease in the extent of activation by ADP was observed. In this case, the modified enzyme is activated 1.3-fold in comparison to a 2.3-fold activation observed for native enzyme.

Glutamate dehydrogenase isolated after modification in the presence of 100 μM GTP and 100 μM DPNH exhibits the same concentration dependence for activation by ADP and the same fold activation as does native enzyme: the curve generated is superimposable on curve A of Figure 6. These results indicate that inclusion of GTP and DPNH in the reaction mixture protects against the effects of modification on the maximum degree of ADP activation as well as on the sensitivity to GTP inhibition. For comparison, inclusion of 2 mM ADP and 100 μM DPNH in the reaction mixture does not protect against the decrease in the maximum extent of activation by ADP, as assayed in the presence of saturating concentrations of ADP. These results suggest that the decrease in the extent of ADP activation observed for modified enzyme is the result of an indirect effect produced by modification at a separate GTP site.

As mentioned previously, glutamate dehydrogenase is inhibited by high concentrations of DPNH by binding to a second site distinct from the catalytic site. Figure 7 shows that the modified and native enzymes have essentially the same affinity for the coenzyme and that both are inhibited by high concentrations of DPNH. These results indicate that 5'-FSBϵA does not react at the DPNH regulatory site.

Binding of GTP by Native and Modified Enzyme. To determine whether 5'-FSBϵA reacts at a GTP site of glutamate dehydrogenase, we measured the reversible binding of [U-^{14}C]GTP to enzyme containing 1.28 mol of 5'-SBϵA incorporated/peptide chain by an ultrafiltraton technique.

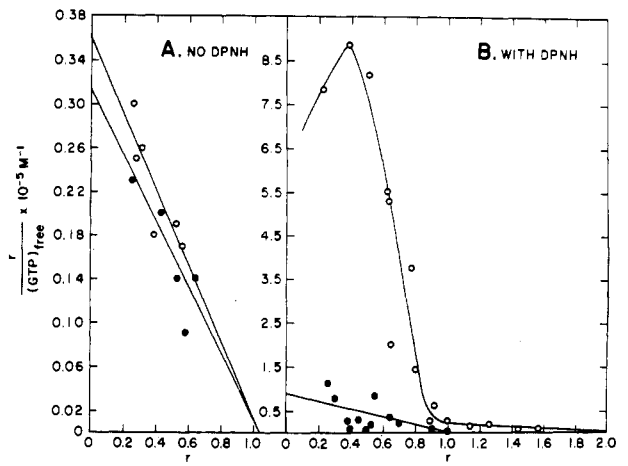

FIGURE 8: Binding of GTP to native and modified glutamate dehydrogenase in Tris–0.044 M acetate buffer (pH 7.1) containing 8.75 mM potassium phosphate and 87.5 μM EDTA. (A) Native enzyme [(O) 1 mg/mL] in the absence of DPNH; modified enzyme [(●) 0.5 mg/mL] containing 1.28 mol of 5'-SBϵA incorporated/subunit, in the absence of DPNH. (B) Native enzyme [(O) 0.5 mg/mL] in the presence of 100 μM DPNH; modified enzyme [(●) 0.5 mg/mL] containing 1.28 mol of 5'-SBϵA incorporated/subunit, in the presence of 100 μM DPNH.

Measurements were made at 25 °C in Tris–0.044 M acetate buffer (pH 7.1) containing 8.75 mM potassium phosphate and 87.5 μM EDTA to maintain the stability of the enzyme. The binding data were analyzed in terms of the Scatchard equation:

$$\frac{r}{GTP_{free}} = \frac{n}{K_d} - \frac{r}{K_d} \qquad (11)$$

where r is the number of moles of GTP bound per peptide chain, n is the number of GTP binding sites per subunit, and K_d is the dissociation constant for the enzyme–GTP complex.

In the absence of DPNH, the binding of GTP to native glutamate dehydrogenase exhibits a linear Scatchard plot extrapolating to 1.02 mol of GTP bound/mol of peptide chain (Figure 8A). A dissociation constant of 28.5 μM is obtained from the slope, a value comparable to that previously reported for native enzyme by Pal & Colman (1979). In the absence of DPNH, the binding of GTP to modified enzyme is similar

to native enzyme (Figure 8A). The Scatchard plot extrapolates to 1.03 mol of GTP bound/mol of peptide chain with a dissociation constant of 33 μM.

For the native enzyme, in contrast to the binding of GTP in the absence of DPNH, 2 binding sites for GTP/peptide chain are observed in the presence of 100 μM DPNH (Figure 8B). Binding to the high-affinity site exhibits a distinct nonlinearity indicative of an apparent cooperativity among the subunits. This is in agreement with results reported by Colman & Frieden (1966). A dissociation constant of 0.6 μM is determined from the limiting slope of the high-affinity site. Binding to a second site is demonstrated when higher concentrations of GTP are utilized. In contrast to the first site, a linear Scatchard plot results from binding of GTP to the second site. The dissociation constant is estimated as 40 μM, a value significantly weaker than that for the first site.

The binding of GTP to glutamate dehydrogenase in the presence of DPNH is dependent on protein concentration and on the state of aggregation of the enzyme (Frieden & Colman, 1967). Since the dissociation constants were previously measured at 0.2 mg/mL and 1.0 mg/mL (Pal & Colman, 1979), the binding to native enzyme was repeated at 0.5 mg/mL so that a direct comparison could be made between native enzyme and modified enzyme. The dissociation constants obtained at 0.5 mg/mL agree with the values determined by Pal & Colman (1979) for native enzyme at 0.2 mg/mL. The minimal effect of this difference in protein concentration on the dissociation constant is reasonable since it has been reported (Frieden & Colman, 1967) that there is little change in molecular weight between 0.5 and 0.3 mg/mL.

The dissociation constant measured in this paper from the kinetic inhibition by GTP of native enzyme is much lower (0.025 μM) than the constant determined by direct binding studies for even the high-affinity GTP site. It is notable that the standard conditions under which the dissociation constant was determined kinetically (Tris–0.01 M acetate buffer, pH 8) differ significantly from those under which the dissociation constant was determined by direct binding (Tris–0.044 M acetate buffer, pH 7.1, containing 8.75 mM potassium phosphate and 87.5 μM EDTA). It has been reported that inorganic phosphate weakens the binding of GTP to glutamate dehydrogenase (Frieden, 1976; Koberstein et al., 1977). The observed difference in the dissociation constants determined by the two methods could possibly be due to the effect of phosphate present in the binding experiments. The dissociation constant for the enzyme–GTP complex was thus determined kinetically with the same buffer as in the binding studies, yielding K_d = 0.2 μM, a value 8-fold higher than that measured at pH 8 in the absence of inorganic phosphate. For comparison, the GTP dissociation constant of 0.6 μM measured by direct binding is only 3-fold higher than the kinetically determined value of 0.2 μM measured in the same buffer.

In contrast to the native enzyme's ability to bind 2 mol of GTP in the presence of DPNH, only 1 mol of GTP is bound by the modified enzyme (Figure 8B) even at concentrations of GTP as high as 100–150 μM (levels at which the second low-affinity GTP site is revealed for the native enzyme). An approximately linear Scatchard plot was observed for the modified enzyme, which extrapolates to 0.97 mol of GTP bound/mol of peptide chain. These results indicate that, upon modification of glutamate dehydrogenase by 5′-FSBεA, there is a loss of the apparent cooperativity among subunits observed in the native enzyme, in addition to the elimination of one GTP binding site. The dissociation constant for the residual GTP site is estimated to be 11.7 μM, a value 20 times greater than the high-affinity GTP site observed for native enzyme in the presence of DPNH.

The K_d for modified enzyme measured from the direct GTP binding experiments is approximately 32-fold weaker than the constant measured kinetically from the inhibition by GTP of the modified enzyme under the standard conditions at pH 8 in the absence of inorganic phosphate. A dissociation constant of 1 μM was measured kinetically in the same buffer utilized in the binding studies, but this value is still approximately 12-fold lower than the dissociation constant measured by direct binding studies.

There are two possible explanations for the discrepancy in the values obtained kinetically and by direct binding. The state of polymerization plays an important role in the binding of GTP to glutamate dehydrogenase in the presence of DPNH (Frieden & Colman, 1967). Thus, it is conceivable that a smaller dissociation constant would be observed kinetically where protein concentrations are low in comparison to direct binding measurements. The observed differences may also be due to an effect on GTP binding produced by added substrates (i.e., α-ketoglutarate and NH_4^+) not present in the binding studies but necessary for kinetic determinations of the dissociation constant.

Discussion

The fluorescent nucleotide analogue 5′-[*p*-(fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine reacts covalently with glutamate dehydrogenase and exhibits the characteristics of an affinity label of a GTP inhibitory site of the enzyme. The compound reacts in a limited and specific manner with 1.28 mol of 5′-SBεA incorporated/mol of subunit. The inclusion of GTP in combination with DPNH in the reaction mixture protects against reaction. In addition to these properties, the rate of reaction as a function of 5′-FSBεA concentration follows saturation kinetics. These observations are consistent with the accepted criteria for an affinity label.

The function of the site modified as a result of reaction with 5′-FSBεA can be ascertained from the kinetic and binding properties of the modified enzyme in combination with the pattern of protection against modification afforded by various ligands involved in the catalytic and regulatory mechanism of the enzyme. The catalytic site does not appear to be affected by 5′-FSBεA since incubation with the reagent caused no loss in enzymatic activity as measured in the absence of regulatory compounds. Furthermore, inclusion of the substrates α-ketoglutarate and DPNH in a reaction mixture does not protect against the modification of glutamate dehydrogenase by 5′-FSBεA.

It is possible that one the regulatory sites on the enzyme is the target of modification by 5′-FSBεA. The rate constant for the reaction of 5′-FSBεA with glutamate dehydrogenase is not affected by high concentrations of DPNH. In addition, the modified enzyme retains the ability to be inhibited by high concentrations of DPNH. These results contrast with the affinity labels 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine and 3′-[*p*-(fluorosulfonyl)benzoyl]adenosine, which have been shown to modify the DPNH inhibitory site of glutamate dehydrogenase. It is concluded that 5′-FSBεA reacts differently from these other affinity labels and does not modify the DPNH inhibitory site.

The most striking feature of the reaction of 5′-FSBεA with glutamate dehydrogenase is the desensitization, but not complete elimination, of GTP inhibition: the maximum extent of inhibition is only 70% for modified as compared with 96% for native enzyme, and K_{GTP} is increased to 0.37 μM, approximately 15 times higher than that for native enzyme (0.025

μM). Complete protection against modification can be produced by including GTP together with DPNH in the reaction mixture. These results suggest that 5′-FSBεA is reacting at a GTP inhibitory site on glutamate dehydrogenase. The most direct evidence for the proposed site of modification is obtained from the binding of GTP to modified enzyme in comparison to native enzyme. Modification results in the elimination of one GTP site as measured in the presence of DPNH. In addition, there is a loss in the apparent cooperativity among the subunits for the binding of GTP in comparison with that observed for native enzyme.

The site of modification can be deduced from a comparison of the binding and kinetic characteristics exhibited by the residual GTP site of the modified enzyme with the properties of the two GTP sites normally observed in the native enzyme in the presence of DPNH. The following reasoning indicates that the higher affinity GTP site is modified as a result of reaction of 5′-FSBεA with glutamate dehydrogenase. The GTP site remaining after modification resembles the native enzyme's low-affinity site in that no cooperativity for the binding of GTP is observed. Furthermore, the rate of modification of the enzyme can be most readily affected by including in the incubation mixture DPNH together with GTP (5–25 μM) in a concentration range expected to bind at the high-affinity site (K_d = 0.6 μM for native enzyme). If reaction were occurring at the low-affinity site, DPNH in combination with much higher concentrations of GTP (>40 μM) would be required to produce an alteration in the reaction rate constant. The dissociation constant of 11.7 μM determined for the GTP site of the modified enzyme is a value that is not similar to the dissociation constant of 0.6 μM or 40 μM for the high- and low-affinity GTP sites of the native enzyme. It can be postulated that the two GTP sites observed in the presence of DPNH are not totally independent but rather are coordinated in their binding of GTP. Thus, modification of one of the GTP sites, in this case the high-affinity GTP site, could cause a perturbation in the properties of the remaining weaker affinity site. The observed difference between the measured dissociation constants of modified and native enzymes could be the result of the modification at the high-affinity GTP site affecting the remaining low-affinity GTP site by increasing its affinity for GTP. Thus, the dissociation constant of 11.7 μM would be a smaller value in comparison to the constant determined for native enzyme. Alternatively, on the basis of the above argument concerning the interaction of the two GTP sites observed in the presence of DPNH, it can be proposed that modification occurs at the low-affinity GTP site, causing a decrease in the affinity for GTP at the originally high-affinity site concomitant with a loss in the apparent cooperativity among the subunits for the binding of GTP. However, this postulate is inconsistent with the concentrations of GTP (in the presence of DPNH), which decrease the rate constant for modification by 5′-FSBεA.

The modification by 5′-FSBεA appears to affect indirectly the extent of activation of glutamate dehydrogenase by ADP. The rate constant for GTP desensitization is slightly lower in the presence of ADP alone or in combination with DPNH. However, the covalent incorporation measured as a function of percent change in GTP inhibition is in agreement with that expected for a limited modification produced in the presence of these partially protecting ligands. If reaction were occurring both at a GTP and at an ADP site, the inclusion of ADP and DPNH might have been expected to protect specifically against reaction at an ADP site, resulting in an enzyme that was normal in its response to ADP; this expectation is not realized. These observations imply that the ADP site is affected indirectly as a result of modification at a separate GTP site. These results are in agreement with previous evidence indicating an interaction between the ADP and GTP sites. It has been shown that ADP competes kinetically with GTP and can overcome the inhibitory effect produced by the guanine nucleotide (Goldin & Frieden, 1972). On the other hand, several studies have shown that the two sites can be independently affected as a result of chemical modification (Colman & Frieden, 1966; Goldin & Frieden, 1971). As a result, it has been suggested that the regulatory nucleotides ADP and GTP bind at different sites with the possibility that these sites overlap either physically or as a result of a ligand-induced conformational change.

As indicated above, the extent of modification of glutamate dehydrogenase by 5′-FSBεA is limited to about 1 mol/peptide chain. It has been shown, in contrast to reaction of other fluorosulfonyl derivatives with certain other enzymes, that dithiothreitol has no effect either on the kinetics of the reaction or on the incorporation of the reagent by the enzyme. These results suggest that a cysteine residue is not the target of 5′-FSBεA in glutamate dehydrogenase. The type of amino acid modified by 5′-FSBεA in this enzyme has not yet been identified; however, lysine and/or tyrosine residues of several proteins have been shown to be modified by the fluorosulfonyl class of nucleotide analogues (Annamalai & Colman, 1981; Esch & Allison, 1978; Likos et al., 1980; Weng et al., 1980; Saradambal et al., 1981). Studies are in progress to identify the amino acid residue modified in glutamate dehydrogenase.

The consequences of modifying various positions in the base moiety and/or in the polyphosphate chain of purine nucleotides in relation to the effects on glutamate dehydrogenase were examined by Lascu et al. (1977). In this report, it was concluded that the inhibitory GTP site was not very specific in its requirement for structural integrity of the purine nucleotide effector and would accept many natural and modified nucleoside triphosphates as inhibitors. However, the activating ADP site exhibited a much higher specificity for purine nucleotides, requiring the naturally occurring ADP as an activator. The potential for 5′-FSBεA acting as a guanine nucleotide analogue is consistent with these results. As shown for εATP (Lascu et al., 1977) as well as for εADP, the modification of the N-1 and 6-NH_2 positions of the adenine ring of the nucleotide leads to the recognition by glutamate dehydrogenase as an inhibitory guanine nucleotide. The C-6/N-1 amide portion of the purine ring has been implicated as being involved in binding to the GTP inhibitory site. The etheno portion of the modified purine ring may approximate the area occupied by the C-6/N-1 region of the purine ring and, lacking the free C-6 amino group, be perceived by the enzyme as a guanosine analogue rather than an adenosine analogue. The inhibitory effects produced by εATP and εADP and, correspondingly, the reaction of 5′-FSBεA at a GTP site may be the result of a lower degree of specificity in nucleotide structure required by the GTP inhibitory site as compared with the other regulatory sites. Additional support for the enzyme's ability to recognize the etheno derivative of adenosine as a guanine nucleotide is provided by a comparison of 5′-FSBεA with the guanine nucleotide affinity label 5′-[*p*-(fluorosulfonyl)benzoyl]guanosine (Pal & Colman, 1979). This compound reacts similarly to 5′-FSBεA in that the elimination of a GTP site is observed as a result of covalent modification; however, in that case 2.3 mol of the reagent is incorporated per enzyme subunit. It was determined that only 1 mol of reagent is incorporated at a GTP site, while a second mole

reacts at a nonspecific site affecting ADP activation. The results reported in this paper suggest that 5′-FSBεA may be a more limited and specific affinity label for a GTP site on glutamate dehydrogenase. Having localized the functional site at which 5′-FSBεA modifies glutamate dehydrogenase, we are now in a position to use this covalent fluorescent probe to monitor conformational changes and measure distances between sites on the enzyme; such studies are in progress.

Acknowledgments

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References

Annamalai, A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 10276–10283.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Brand, L., & Witholt, B. (1967) *Methods Enzymol. 11*, 776–856.

Colman, R. F., & Frieden, C. (1966) *J. Biol. Chem. 241*, 3652–3660.

Colman, R. F., & Foster, D. S. (1970) *J. Biol. Chem. 245*, 6190–6195.

Dieter, H., Koberstein, R., & Sund, H. (1974) *FEBS Lett. 47*, 90–93.

Esch, F. S., & Allison, W. S. (1978) *J. Biol. Chem. 253*, 6100–6106.

Frieden, C. (1959) *J. Biol. Chem. 234*, 809–814, 815–820.

Frieden, C. (1963) *J. Biol. Chem. 238*, 3286–3299.

Frieden, C. (1976) *The Urea Cycle (Conference)* (Grisolia, S., Baguena, R., & Mayor, F. Eds.) p 59, Wiley, New York.

Frieden, C., & Colman, R. F. (1967) *J. Biol. Chem. 242*, 1705–1715.

Goldin, B. R., & Frieden, C. (1971) *Biochemistry 10*, 3527–3533.

Goldin, B. R., & Frieden, C. (1972) *Curr. Top. Cell. Regul. 4*, 77–117.

Horton, H. R., & Koshland, D. E. (1967) *Methods Enzymol. 11*, 856–870.

Jacobson, M. A., & Colman, R. F. (1981) Abstracts of the American Chemical Society Division of Biological Chemistry, Minneapolis, MN.

Koberstein, R., Dieter, H., Narkau, K., & Sund, H. (1977) 2nd International Symposium on Pyridine Nucleotide Dependent Dehydrogenases, Konstanz, West Germany.

Lascu, I., Bârzu, T., Ty, N. G., Ngoc, L. D., Bârzu, O., & Mantsch, H. H. (1977) *Biochim. Biophys. Acta 482*, 251–260.

Likos, J. J., & Colman, R. F. (1981) *Biochemistry 20*, 491–499.

Likos, J. J., Hess, B., & Colman, R. F. (1980) *J. Biol. Chem. 255*, 9388–9398.

Olson, J. A., & Anfinsen, C. B. (1952) *J. Biol. Chem. 197*, 67–79.

Pal, P. K., & Colman, R. F. (1979) *Biochemistry 18*, 838–845.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975a) *J. Biol. Chem. 250*, 8140–8147.

Pal, P. K., Wechter, W. J., & Colman, R. F. (1975b) *Biochemistry 14*, 707–715.

Pantaloni, D., & Dessen, P. (1969) *Eur. J. Biochem. 11*, 510–519.

Penefsky, H. S. (1979) *Methods Enzymol. 56*, 527–530.

Saradambal, K. V., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 11866–11872.

Secrist, J. A., Barrio, J. R., Leonard, N. J., & Weber, G. (1972) *Biochemistry 11*, 3499–3506.

Smith, E. L., Landon, M., Piskiewicz, D., Brattin, W. J., Langley, J. J., & Malamed, M. D. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 724–730.

Stryer, L. (1978) *Annu. Rev. Biochem. 47*, 819–846.

Togashi, C. T., & Reisler, A. L. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1786.

Tomich, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.

Weng, L., Henrikson, R. L., & Mansour, T. E. (1980) *J. Biol. Chem. 255*, 1492–1496.

Williamson, J. M. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1866.

Yanagida, T. (1981) *J. Mol. Biol. 146*, 539–560.